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Phylogenetic group determination of *Escherichia coli* isolated from broilers and layers with colibacillosis

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Abstract

Colibacillosis is of the most common infectious bacterial diseases of poultry.A total of 170 *Escherichia coli* isolates obtained from broiler and layer flocks implicated with colibacillosis between 2011 and 2014 were subjected to phylogenetic analysis. Among 150 *E. coli* isolates from typical lesions of local and systemic colibacillosis, 54 (31.8%), 37 (21.7%), 36 (21.2%) and 43 (25.3%) isolates determined as belonged to groups A, B1, B2 and D, respectively. The distribution of phylogenetic types for 20 isolates, obtained from apparently healthy birds as controls, were 9 (45%), 5 (25%), 1 (5%) and 5 (25%) for A, B1, B2 and D, respectively. Overall, the phylogenetic Determination revealed the B2 groups as predominant isolates in diseased birds, whereas the A group was apparently predominant in healthy birds. Results of this study represent genotypic diversity among different manifestations of avian colibacillosis.

Keywords: Colibacillosis, *Escherichia coli*, phylogenetic types and broiler and layer flocks

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Introduction

Escherichia coli is a bacterium widely distributed among warm-blooded animals and known as a normal inhabitant of the gut microflora (David E. Swayne 2013). Escherichia coli is a multi-talented, enteric Gram-negative bacillus and best known as non-invasive commensal that grow in mass culture in the human as well as in animal gut lumen, perhaps prohibiting other more harmful bacteria from proliferation. Airsacculitis, cellulitis, pericarditis, perihepatitis and respiratory distress are among the most commonly associated signs of colibacillosis (Nolan *et al*., 2013). Escherichia coli strains can be classified to one of the four main phylogenetic groups A, B1, B2 and D which is the basis of phylogenetic studies of the species (Clermont *et al*., 2000). These four phylogenetic groups can be distributed into seven subgroups (A0, A1, B1, B22, B23, D1, D2) according to the combination of the three genetic markers chuA, yjaA and an anonymous DNA fragment TspE4.C2 (Carlos et al., 2010; Clermont *et al*., 2013). The *chuA* is a gene involved in heme transport in enterohemorrhagic O157:H7, and *yjaA*is known to be involved in cellular response to hydrogen peroxide and acid stress. The function of TspE4.C2 is not yet understood (Lee, 2011). Each strain of four groups has various phenotypic features, causing their ability to exploit different sugars, antibiotic resistance profiles and growth rate-temperature relationships (Herzer *et al*., 1990). Several studies have shown the relationship between phylogeny and pathogenicity of *E. coli* strains (Bashir S, 2012; E. Bingen *et al*., 1998; Escobar-Paramo *et al*., 2004; Picard *et al*., 1999). One study have stated that most commensal *E. coli* strains belong to A and B1 groups (Duriez *et al*., 2001); while the virulent extraintestinal *E. coli* strains mainly belong to group B2 and to a lesser extent to group D (Picard, et al., 1999). Several techniques can be performed to determine phylogenetic group, such as multilocus enzyme electrophoresis (Herzer, *et al*., 1990; Selander *et al*., 1986), ribotyping (E. Bingen, *et al*., 1998; E. H. Bingen *et al*., 1994), random amplified polymorphic DNA analysis (Desjardins *et al*., 1995), fluorescent amplified-fragment length polymorphism (FAFLP) analysis (Desjardins, *et al*., 1995), PCR phylotyping by using the presence/absence of three genomic DNA fragments (Clermont, *et al*., 2000), analysis of variation at mononucleotide repeats in intergenic sequences (Diamant *et al*., 2004), and multilocus sequence typing (MLST) (Lecointre et al., 1998; Reid *et al*., 2000). Clearly, MLST method is now the “gold standard” technique (Urwin& Maiden, 2003), but it is complex and time-consuming and also requires a collection of typed strains. The method registered by Clermont *et al*. (2000), whose results strongly correlate with those obtained by other standard methods is an excellent technique for rapid and inexpensive assigning of E. coli strains in various phylogenetic groups (Clermont, *et al*., 2000; Gordon et al., 2008). Majority of E. coli strains that are able to persist in the environment belong to the B1 phylogenetic group (Walk *et al*., 2007). Furthermore, genome size differs between these phylogroups, with A and B1 strains having smaller genomes compared to B2 or D strains (Bergthorsson&Ochman, 1998) and strains from phylogroups B2 and D have more virulence factors than strains from the phylogroups A and B1 (J. R. Johnson *et al*., 2001). Characterization of phylogenetic groups depends on clinical interest, as group A and B1 are generally associated with commensal strains whereas most enteropathogenic isolates are attributed to group D, and group B2 is associated with extra-intestinal pathotypes (Lay *et al*., 2012).

Up to now, there have been very few published studies on phylogenetic grouping of avian pathogenic *E. coli* (APEC) in Iran and other countries and none has compared *E. coli* phylogenetic distribution among various *E. coli*-associated disease manifestations in broilers and layers. Herein, we report the phylogenetic group determination of 170 *E.*

coli isolated from chickens in various parts of Iran.

Materials and methods

*Sampling procedure***:** A total of 170 *E. coli* isolateswere used in this study, from which 150 isolates were recovered from different organs of broilers (126 isolates) and layers (24 isolates) with lesions typical of colibacillosis such as pericarditis and perihepatitis (PP), swollen head syndrome (SHS), infected yolk sac (YSI), inflamed infraorbital sinus and head femoral necrosis (HFN). Twenty faecal samples were also obtained from apparently healthy birds and used as control isolates in this study. Random samples were collected from selected flocks located throughout Iran deposit in Tehran and Mashhad University's bacterial colection.

Culture and biochemical characterization: In order to confirm *E. coli* isolates, visceral organs such as the liver, heart, yolk sac, infraorbital sinus and bone marrow swabs were cultured onto MacConkey (MC) agar (Merck, Germany) and incubated aerobically at 37°C for 24 hours. Suspected *E. coli* colonies such as faecal swabs were subsequently inoculated onto Eosin-Methylene blue (EMB) agar plates (Titrachem, Iran) and incubated at the same time and temperature as described previously. The recognition of *E. coli*was based on the results of diagnostic tests, which included Gram stain, catalase and oxidase tests (Quinn *et al*., 1994).

DNA Extraction: DNA template preparation was performed by the boiling method as follows. Briefly, a few colonies of each pure *E. coli* isolate grown on blood agar plate at 37°C for 24 hours were picked up and suspended in 250 µl TE buffer. The cells were lysed by heating at 95°C for 15 min. After heating, the lysed cells were immediately were put in refrigerator for 5 min. The supernatant was then harvested by centrifugation at 10000 *g* x for 5 minut, transferred to a fresh microcentrifuge tube and kept at -20°C for future use. The supernatant was used as a source of template for amplification (Abdallah *et al.*, 2011).The concentration of DNA was determined by NanoDrop 2000 (Thermo Fisher Scientific, USA) and adjusted to approximately 50 ng/µl for each PCR reaction.

Multiplex Polymerase Chain Reaction (M-PCR): Phylogenetic assignment of *E. coli* isolates was done using a previously reported Triplex PCR-based phylotyping technique (Clermont, et al., 2000; Gordon, et al., 2008). Briefly, the genomic DNA of each *E. coli* isolate was amplified by triplex PCR by using primers targeted at three markers *chuA*, *yjaA*and TspE4.C2. The primer sequences described in Table 1 were synthesized by SinaClon (Iran) and other materials used in PCR reaction were provided from Ampliqon (Denmark). Amplification reactions were carried out in a 25 µl reaction volume containing 2.5 µl 10 x PCR buffer (supplied with *Taq*DNApolymerase), 1 µldNTP (200 µM each of dATP, dCTP, dGTP, and dTTP), 1 µl (20 pmol) of each primer, 2.5 U of *Taq*DNApolymerase, 0.75 µl (… mM) $MgCl_2$, and 11.25 µl dH_2O. Approximately 5 ng of template DNA (3 µl) was added to the mixture. In all PCR reaction sets, negative controls (dH_2O instead of template DNA) were included. Amplification was programmed in a thermocycler (TECHNE TC-3000, UK) as follows: 94° C for 4 min followed by 30 cycles of 94° C for 5 s, 59° C for 10 s, 72° C for 2 min, and a final extension at 72° C for 5 min.

The amplification products were detected by gel electrophoresis in 2% agarose gel at 120 V for 60 min in 1 x TAE buffer.

Statistical analysis

Frequency of phylogenetic groups between the studied groups was compared by chi-square andFisher's exact test. Abundance of group A in assumed healthy birds category was significantly higher than the YSI group (p<0.001), group A frequency in SHS category was higher than YSI group (p= 0.009), plentifulness of group A in pericarditis

and perihepatitis category was higher than YSI group (p = 0.001) and finally frequency of group A in HFN category was higher than YSI group (p = 0.046). It should be noted that abundance of group B1 between these six groups did not show any significant difference. Group B2 frequency in isolates provided from layers was higher than assumed healthy birds group (p = 0.022), aboundness of group B2 in YSI category was higher than assumed healthy birds group (p = 0.015), in YSI Category frequency of group B2 was higher than Pericarditis and perihepatitis isolates of broilers (p=0.039) and finally plentifulness of group B2 in *E.coli* isolates of layers was higher than HFN group (p = 0.04). Ampleness of group D in SHS category was higher than Pericarditis and perihepatitis cases of broilers (p = 0.008) and also group D frequency in YSI category was higher than pericarditis and perihepatitis cases of broilers (p = 0.04).

Results

Among 170 isolates, 54 (31.8%), 37 (21.7%), 36 (21.2%) and 43 (25.3%) isolates belonged to groups A, B1, B2 and D, respectively (Table1).

Table 1.Prevalence of *E. coli* isolates belong to each four main phylogenetic groups according to three biomarkers.

Phylo-groups	Layers (PP)	Broilers					Total
		Healthy	PP	YSI	SHS	HFN	
A	5(20.8%)	9(45%)	24(41.3%)[b]	-[a]	7(41.2%)[b]	9(25.7%)[b]	54(31.7%)
B1	6(25%)	5(25%)	15(25.9%)	1(6.2%)	-	10(28.6%)	37(21.6%)
B2	4(16.7%)[c,f]	5(25%)	8(13.8%)	8(50%)[d,e]	7(41.2%)	11(31.4%)	43(25.2%)
D	9(37.5%)	1(5%)	11(19%)	7(43.8%)[g]	3(17.6%)[g]	5(14.3%)	36(21%)
Total	24	20	58	16	17	35	171

[a] Represents significant difference between (healthy and YSI), [b] (SHS and YSI), [c] (PP and YSI), [d] (layers and healthy), [e] (YSI and PP, healthy), [f] (layer and HFN) and [g] (PP and SHS,YSI)

Among a total of 126 *E. coli* isolates from broilers, group A and B1 contained the majority of the collected isolates (40 isolates, 31.74% and 26 isolates, 20.63%) respectively, followed by group D (34 isolates, 26.98%) and finally group B2 contained 26 isolates (20.63%) (Fig.1). Group distribution of the isolates obtained from assumed healthy birds for control group was as follows: A (9 isolates, 45%), B1 (5 isolates, 25%), B2 (1 isolate, 5%) and D (5 isolates, 25%) (Fig.2). Classification and frequency of the 24 *E. coli* isolates from layers birds with egg peritonitis, pericarditis and perihepatitis was as follows: A (5 isolates, 20.83%), B1 (6 isolates, 25%), B2 (9 isolates, 37.5%) and D (4 isolates, 16.66%) (Fig. 3).

Discussion

Escherichia coli is, entirely or partly, the cause of a variety of disease manifestation in poultry (Nolan *et al*. 2013). *Escherichia coli* isolated from such disease manifestation have been assigned to four main phylogenetic groups (Herzer, et al., 1990), each of them represent ecological specialization and differ in their tendency to cause disease (Walk, et al., 2007). Hence, knowledge of the structure of bacterial populations is a prerequisite to perception the epidemiology of infectious disease. Nowadays, there is an increasing evidence that virulence in extraintestinal *E. coli* (ExPEC) infections is more likely linked to the phylogenetic background of a strain than to its ecological background (Moulin-Schouleur *et al*., 2007; Picard *et al*., 1999). The common phylogenetic origins of APEC and other ExPEC, emphasizes on the potential zoonotic risk of APECs (T. J. Johnson *et al.*, 2007; Moulin-Schouleur, et al., 2007; Wirth *et al.*, 2006). Phylogenetic analysis of human isolates of *E. coli* has shown that these *E. coli* are composed of four main phylogenetic groups A, B1, B2 and D. The commensal *E. coli* are usually associated with groups A and B1 but the ExPEC and enteropathogenicpathotypes are usually allocated to group B2 and D,

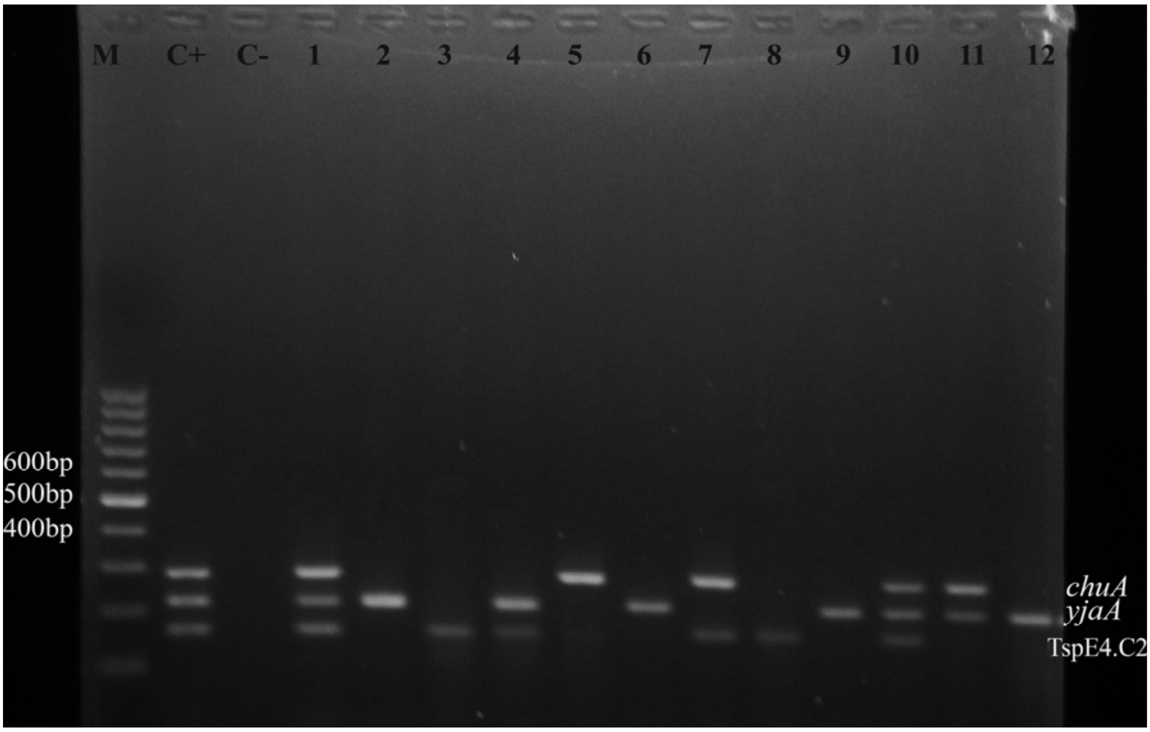


Figure 1. Presence of the three biomarkers in E. coli isolates obtained from pericarditis and perihepatitiscasese of broilers was determined using triplex PCR. Marker (100 bp ladder), positive and negative control are located before lane 1. Lanes 2, 6, 9 and 12, group A; Lanes 3,4 and 8, group B1; Lanes 5 and 7, group D; Lanes 1, 10 and 11, group B2.

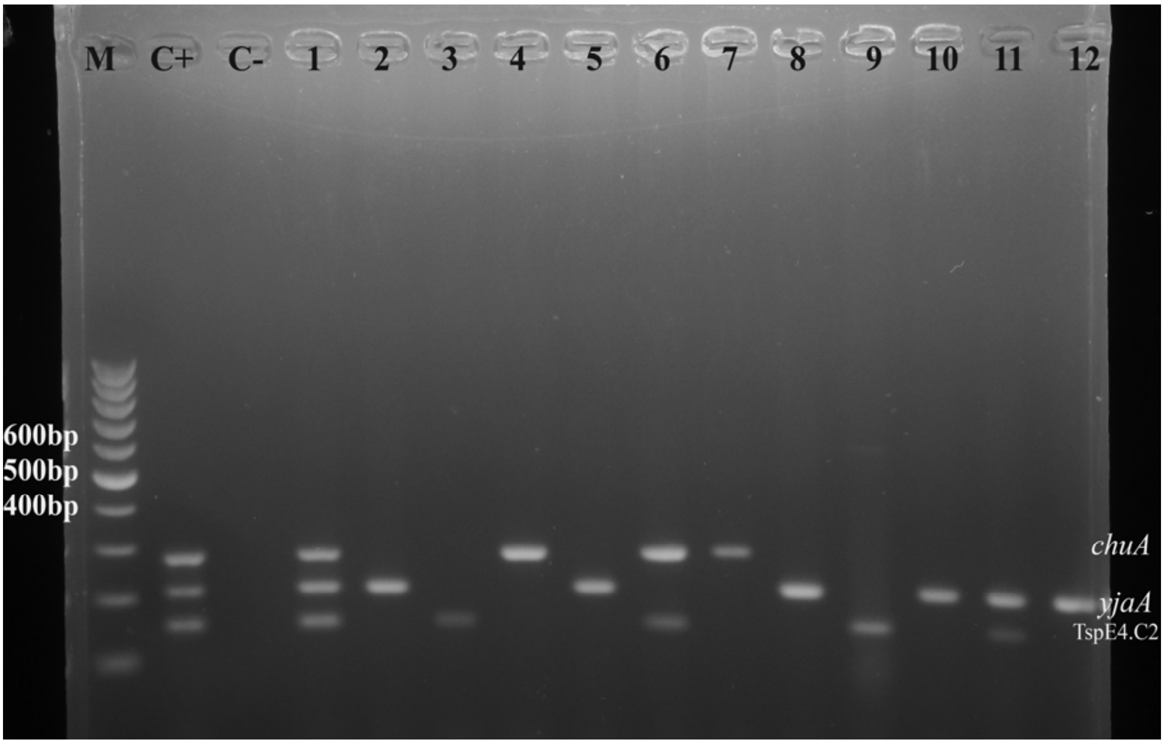


Figure 2. Representative gel of triplex genotyping PCR assay conducted on *E. coli* originating from fecal samples of poultry apparently healthy. Each combination of *chuA*and *yjaA*gene and DNA fragment TspE4.C2 amplification created phylogenetic group determination of a strain. Marker (100 bp ladder), positive and negative control are located before lane 1. Lanes 2, 5, 8, 10 and 12, group A; Lanes 3, 9 and 11, group B1; 4, 6 and 7, group D; Lane 1, group B2.

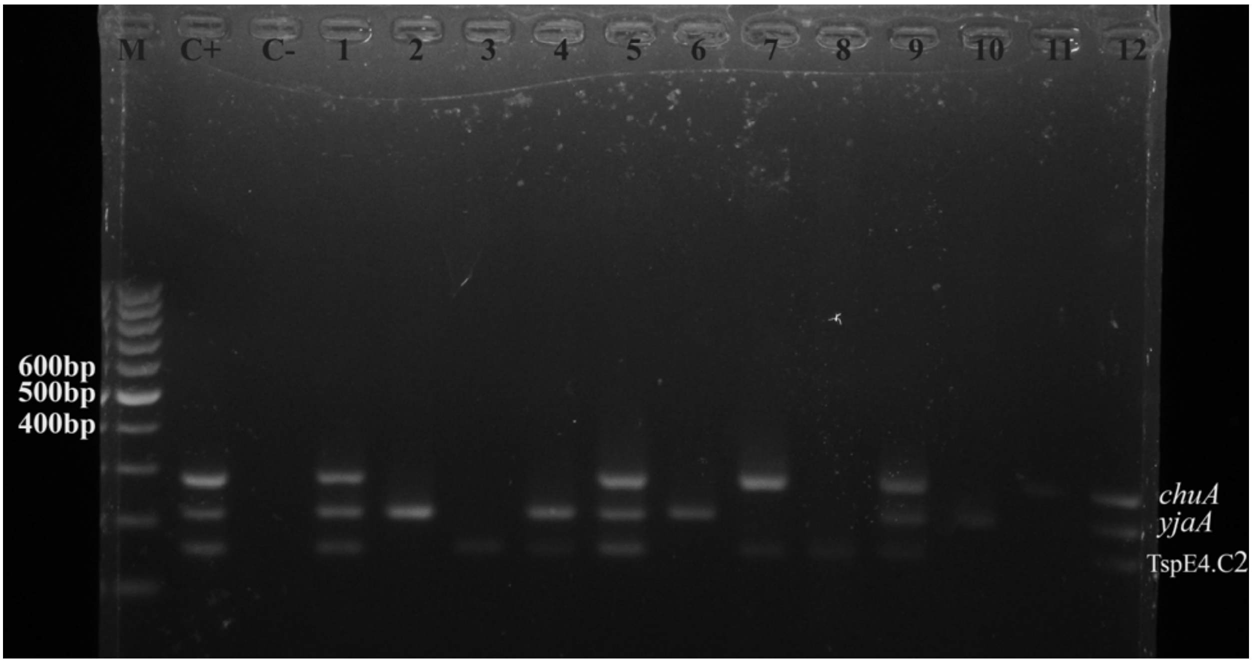


Figure 3. The presence of *chuA*(279 bp), *yjaA*(211 bp) and TspE4.C2 (152 bp) in *E. coli* isolates obtained from percicarditis, perihepatitis and peritonitis cases of layer carcasses was determined using multiplex colony PCR. Marker (100 bp ladder), positive and negative control are located before lane 1. Lanes 2, 6 and 10, group A; Lanes 3, 4 and 8, group B1; Lanes 7 and 11, group D; Lanes 1, 5, 9 and 12, group B2.

respectively (Sabarinath A, 2011). This scheme has been used for the characterization of *E. coli* strains from poultry. In the present study, Phylogroups A and B1 were observed in 70% of the commensal isolates from healthy birds that supports previous findings that phylogroups A and B1 are generally associated with commensals (Asai *et al.*, 2011; Carlos, et al., 2010; Sabarinath A, 2011).

In this study, we also determined phylogenetic groups *E. coli* isolates form diseased commercial layer chickens showing pericarditis and perihepatitis lesions. Most of *E. coli* isolates belonged to group B2 (37.5%) that differed from the findings of Salehi and Ghanbarpour (2010), in which they reported the presence of phylogenetic groups A (41.32%), D (33.88%), B2 (14.87%) and B1 (9.91%) among *E. coli* isolates from salpingitis. In another study, Trampel *et al*. (2007) noted that *E. coli* isolates from laying hens mostly belonged to A and D phylogenetic groups but Dissanayake *et al*. (2008) showed that APEC isolates belonged to A (71%), B1 (4.1%), B2 (7.9%) and D (18.65%) groups.

Ewers *et al*. (2009) compared the phylogenetic types of *E. coli* originating form diseased and healthy birds, and environment based on virulence genotyping and phylogenetic data such as EcoR analysis and MLST data. Their data showed that certain non-outbreak strains originating from the intestine of clinically healthy poultry had zoonotic potential and could be transferred directly from birds to humans or could serve as a genetic pool for ExPEC strains.

In our study, we have chosen isolates from different manifestations of APECs infection in broilers such as swollen head syndrome (SHS), yolk sac infection (YSI), pericarditis and perihepatitis (PP) and head femoral necrosis (HFN) to compare the relevant phylogenetic patterns. A=D>B2>B1 pattern was found in SHS isolates while D>B2>B1>A, A>B1>B2>D and D>B1>A>B2 patterns were belonged to YSI, PP and HFN isolates, respectively.

Rodriguez-Siek*et al.* (2005) reported

phylogroups A (38.0%), D (28.1%), B2 (18.5%) and B1 (15.5%) among APEC isolates and Ewers *et al*., (2009) showed that considerable portions of APEC strains fell into groups A (46.1%) and B2 (35.1%).

A previous study has shown that group A and group D were predominant in APEC in Japan (Moulin-Schouleur *et al.*, 2007) and the United States (T. J. Johnson *et al*., 2007). In another report, PCR assays showed that the isolates fell into the four phylogenetic groups A (41.32%), D (33.88%), B2 (14.87%) and B1 (9.91%) (Asai *et al*., 2011). According to another study, 86 Korean APEC isolates were divided into different phylogenetic groups. Group A was the largest (39.5%, 34/86), groups B1 (23.3%, 20/86) and B2 (22.1%, 19/86) were similar in size, and group D (15.1%, 13/86) was notably smaller Pathotyping APECs in Korea. Phylogenetic analyses of *E. coli* isolates from cellulitis revealed the isolates belonged to phylogroups A (51.56%), B1 (18.75%) and D (29.68%) (Ghanbarpour*et al*., 2009).

In this study, more than 21% of the isolates either isolated from healthy or diseased birds belonged to B2 phylogroup. This phylogroup is closely related to human ExPEC and is frequently found among human uropathogenic and neonatal meningitis *E.coli*strains (E. Bingen *et al.*, 1998; Johnson TJ, 2008). Therefore, further studies on the correlation between B2 group of APEC and human ExPEC isolates may be required to examine the zoonotic potential of these isolates.

In conclusion, the proportion of the phylogenetic groups might be different between APEC and *E. coli* from healthy broilers and we need to do more investigation to find a relationship between the Clermont phylo-groups and the pathogenecity of APECs in birds.

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تعیین گروه فیلوژنتیکی جدایه های اشریشیا کلی از موارد کلی باسیلوز طیور گوشتی و تخم گذار

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چکیده

کلی باسیلوز یکی از شایع ترین بیماری های باکتریایی صنعت طیور است. به منظور آنالیز فیلوژنتیکی، تعداد ۱۷۰ جدایه اشریشیا کلی از گله های طیور گوشتی و تخم گذار مبتلا به کلی باسیلوز در طی سال های ۱۳۸۹ تا ۱۳۹۲ جمع آوری گردید. از تعداد ۱۵۰ جدایه تهیه شده از جراحات مشخص موضعی و سیستمیک کلی باسیلوز به ترتیب ۵۴ (۳۱/۸٪)، ۳۷ (۲۱/۷٪)، ۳۶ (۲۱/۲٪) و ۴۳ (۲۵/۳٪) جدایه متعلق به گروه های A، B1، B2 و D بود. همچنین پراکندگی گروه های فیلوژنتیکیدر ۲۰ جدایه تهیه شده از مدفوع پرندگان به ظاهر سالم به عنوان گروه کنترل شامل ۹ (۴۵٪)، ۵ (۲۵٪)، ۱ (۵٪) و ۵ (۲۵٪) جدایه بود که به ترتیب متعلق به گروه های A، B1، B2 و D می باشد. بنابراین گروه های فیلوژنتیکی B2 و A به ترتیب در بین جدایه های جمع آوری شده از پرندگان مبتلا به کلی باسیلوز و پرندگان به ظاهر سالم غالب بودند. نتایج این مطالعه نشان دهنده تنوع ژنوتیپی در میان انواع مختلف رخدادهای کلی باسیلوز طیور می باشد.

واژگان کلیدی: کلی باسیلوز، گروه های فیلوژنتیکی، جدایه، طیور